Isolation and Characterization of a Succinimide Variant of Methionyl Human Growth Hormone*

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Deamidation of asparagine and glutamine residues, isomerization of aspartic acid side chains, and racemization of the L- to the d-form of the amino acids are common spontaneous chemical reactions known to occur in proteins. Previous studies have implicated succinimides as intermediates in these reactions; however, the evidence has been indirect. Our results demonstrate, for the first time, the presence of a succinimide intermediate in an intact protein.

The succinimide (cyclic imide) variant was isolated from thermally stressed recombinant methionyl human growth hormone (hGH) by high performance anion-exchange chromatography, further purified by reversed-phase high performance liquid chromatography, and analyzed by tryptic mapping. A later eluting tryptic peptide, compared with the native T12 peptide (residues 128–134, Leu-Glu-Asp-Gly-Ser-Pro-Arg), was analyzed by mass spectrometry (MS). This variant had a protonated molecular mass of 755.3 atomic mass units (u), as compared with 773.3 u for the native T12 peptide. A difference of 18 u, a loss of water, is consistent with the formation of a succinimide intermediate at Asp-130 of methionyl hGH. MS/MS analysis of the cyclic imide-containing peptide verified that the modification occurred at Asp-130. A difference of 18 u was also observed for the intact cyclic imide methionyl hGH variant (22,256 u), as measured by electrospray mass spectrometry, compared with native methionyl hGH (22,256 u).

Proteins can be exposed to conditions during isolation or upon storage that accelerate the rate of deamidation of asparagine or isomerization of aspartate side chains. Deamidation of asparagine residues has been shown to be involved in the inactivation of lysozyme (1), ribonuclease A (2), triosephosphate isomerase (3), and soluble CD4 (4). Isomerization of aspartyl to isoaspartyl results in the addition of an extra carbon into the peptide backbone (5). If this were to alter the secondary and/or tertiary structure, the biological function of the protein could be affected. It has, therefore, become important to develop methods for monitoring levels of deamidation and isomerization in proteins. HPIEC† was investigated since deamidation of an asparagine residue results in the formation of a variant that has a different charge than the native protein. Isomerization of an aspartyl residue to an isoaspartyl residue occurs through a cyclic imide intermediate. The cyclic imide variant has a different charge than the native protein and, therefore, is also amenable to separation by HPIEC.

The generally accepted mechanism of deamidation involves two steps (5). In the first step, regarded as the rate-limiting step, an intramolecular cyclic imide intermediate is formed. The imide is then readily hydrolyzed as resulting in the substitution of a carboxyl group for the amide group in an asparagine residue. Either an α or β-(iso)aspartate will be formed, depending upon which bond to the nitrogen atom in the imide ring is broken. The β-aspartate can also be formed through the isomerization of α-aspartate, although this reaction is much slower (5).

In small peptides, the greatest rates of cyclic imide formation have been observed when the adjacent carboxyl residue is glycine, serine, or threonine (6). In proteins, structure, as it affects the accessibility of the α-nitrogen of the adjacent amino acid to the β-carbonyl of the asparagine or aspartate, also exerts a major influence (7). Methionyl-hGH, a polypeptide of 22,256 daltons, contains an aspartate at position 130 with a neighboring glycine. Since Asp-130 is located in a flexible region of the molecule, based on the porcine growth hormone model (8), it is a likely candidate for isomerization. Johnson et al. (9) used protein carboxyl methyltransferase, an enzyme that specifically methylates isoaspartyl linkages, to determine which sites in Met-hGH are prone to deamidation/isomerization. Formation of isoaspartate was detected at two sites, Asn-149 and Asp-130. Our results are in agreement with those of Johnson et al. (9). In addition, we report on the isolation and characterization of an intact Met-hGH variant containing a succinimide (cyclic imide) at Asp-130.

EXPERIMENTAL PROCEDURES AND RESULTS

A thermally degraded sample of Met-hGH (100 µg) that had been stored at 45 °C for 4 months (see "Experimental Procedures") was analyzed by high performance anion-exchange chromatography using a DEAE-3SW column (Fig. 1). Several peaks of greater acridity were resolved. Most of these have been previously characterized as deamidation products, i.e. monodesamido at Asn-149 (11) and didesamido at Asn-149 and Asn-152 (11, 12). A more basic peak was also observed. It was conjectured to be the cyclic imide variant of methionyl hGH since the intermediate formed from isomerization...
Following additional purification by reversed-phase HPLC (Fig. 2), the more basic component (succinimide variant) was analyzed by tryptic mapping (Fig. 3B). A new peptide, T12b, had a molecular mass of 755.3 compared with 773.3 u for the native T12 peptide (Leu-Glu-Asp-Gly-Ser-Pro-Arg). The difference of 18 u, a loss of water, is consistent with the formation of a succinimide. Tandem MS was used to verify that the loss of 18 u occurred at Asp-130. The collisional-activation-dissociation (CAD) daughter spectra of the variant and native peptides are shown in Fig. 5, A and B, respectively. Multiple losses of glycero...
Succinimide Formation in Human Growth Hormone


SUPPLEMENTARY MATERIAL

ISOULATION AND CHARACTERIZATION OF A SUCCINIMIDE VARIANT OF METHIONYL HUMAN GROWTH HORMONE

by

Glen Teshima1, John T. Sulbst, Victor Lien1, and Eleanor Canova-Davis1

EXPERIMENTAL PROCEDURES (METHODS AND MATERIALS)

PREPARATION OF THERMALLY-DEGRADATED METHIONYL hGH

Methionyl-hGH was lyophilized in sodium phosphate buffer, pH 7.2, and incubated at 45°C for 4 months.

HIGH PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY

HPLC was carried out using a Hewlett-Packard 1050 HPLC system. Methionyl-hGH (100 mg to 1 mg) was loaded onto a Tropinose 150 DEAE-30W column (7.5 x 75 mm) at 45°C equilibrated with 50 mM Na2CO3, pH 5.0. Flow rate was 1.0 ml/min. Protein was eluted with a linear NaCl gradient from 0 to 1 M in 75% (A) and 25% (B) acetonitrile over 20 min. Fractions representing the main peak and the variant were lyophilized and then reconstituted with 2% aqueous trifluoroacetic acid prior to analysis by reversed phase HPLC.

REVERSED-PHASE HPLC

The ion-exchange fractions were loaded onto a TosoHass TSK-DEAE-5W column (4.6 x 250 mm) at 40°C equilibrated with 47% acetonitrile containing 0.1% trifluoroacetic acid and eluted with a linear gradient to 52% acetonitrile over 20 min. The flow rate was 1.0 ml/min and the protein was monitored at 280 nm. Fractions representing the main peak and the variant were collected and dried using a Savant Speed-Vac.

TRYPTIC MAPPING

The dried trypsin-digested protein was loaded onto a Nucleosil C18 column (4.6 x 250 mm) at 37°C and eluted with 33% acetonitrile containing 0.1% trifluoroacetic acid and monitored with a UV absorbance set at 214 nm. The protein was acidified with formic acid. Spectra were obtained using a Fisons Speed-Mass Tryptic Mass Spectrometer. Collisional-activation dissociation (CAD) daughter spectra were also obtained with the BIORAD 3000 (MBF-3000) ion source. Precursor ions of 20 to 150 m/z of peptide were selected by MS-1 at resolution of 1000, the 12C isotope peak was selected by MS-1 at resolution of 1000, and the 12C isotope peak was selected by MS-1 at resolution of 1000. daughter ions were isotopically pure. The selected ions leaving MS-1 were fragmented with argon in a ground glass cell and the product ions were monitored using a Linear Mass Spectrometer (LMS). The mass spectrum was obtained using the LMS hypermass program to calibrate the molecular mass from several multiple-charged ions.

RESULTS

The more basic component from ion-exchange chromatography was further purified by reversed phase HPLC (Fig. 2A). Two major peaks were resolved. Peak I corresponded to the main peak from HPLC and Peak II corresponds to the variant peak from HPLC. Both peaks I and II (Fig. 2A) were analyzed by tryptic mapping (Figs. 3 and 4). The samples were collected with a linear acetonitrile containing trifluoroacetic acid: (A) gradient from 25% to 50%.

Fig. 2. Reversed phase HPLC of ion-exchange fractions of native T12 (residues 128-134) and variant T12 peptides were collected and lyophilized and then reconstituted with 0.1% aqueous tris (HCl) and analyzed by mass spectrometry and had a mass axis of 12.5 kDa. The peptides were further analyzed by Tryptic Mass Spectrometry. The proteins were dissolved in glycerol acidified with formic acid. Spectra were obtained using a Fisons Speed-Mass Tryptic Mass Spectrometer. Collisional-activation dissociation (CAD) daughter spectra were also obtained with the BIORAD 3000 (MBF-3000) ion source. Precursor ions of 20 to 150 m/z of peptide were selected by MS-1 at resolution of 1000, the 12C isotope peak was selected by MS-1 at resolution of 1000. The 12C isotope peak was selected by MS-1 at resolution of 1000 and the 12C isotope peak was selected by MS-1 at resolution of 1000. daughter ions were isotopically pure. The selected ions leaving MS-1 were fragmented with argon in a ground glass cell and the product ions were monitored using a Linear Mass Spectrometer (LMS). The mass spectrum was obtained using the LMS hypermass program to calibrate the molecular mass from several multiple-charged ions.

Supplementary Table

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Fig. 3. Reversed phase HPLC tryptic maps of reversed phase purified Met-His fractions. Tryptic mapping was performed on a 5 mg sample of each peak.
Succinimide Formation in Human Growth Hormone

Table 1

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<th>Cycle</th>
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Fig. 6. Reaction schemes to explain molecular ions present in the CAD daughter spectrum of T12b (covalent model). The ions are labeled according to Birktoft's adaptation of the Houckstath-Kofstad ion designations. 

Scheme I

Scheme II

Scheme III

Fig. 6. Reaction schemes to explain molecular ions present in the CAD daughter spectrum of T12b (covalent model). The ions are labeled according to Birktoft's adaptation of the Houckstath-Kofstad ion designations. 

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